

More complicated than they look,
teeth are actually tiny organs.

Test-Tube Teeth

If tissue engineers
can manufacture living replacement teeth,
they would blaze a trail for engineering larger organs
while leading dentistry into the age of regenerative medicine





By Paul T. Sharpe and Conan S. Young

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We take them for granted until they are gone

or require major repairs. And then the options are grim: do without lost teeth or replace them with inert prosthetic versions. In the Western world, an estimated 85 percent of adults have had some form of dental treatment. Seven percent have lost one or more teeth by age 17. After age 50, an average of 12 teeth stand to have been lost.

In theory, a natural tooth made from the patient's own tissue and grown in its intended location would make the best possible replacement, although such bioengineered teeth have for many years been little more than a dream. Recently, however, progress in understanding how teeth first develop has combined with advances in stem cell biology and tissue engineering technology to bring us close to the realization of biological replacement teeth.

Apart from the potential benefit to people who need new teeth, this research also offers two significant advantages for testing the concept of organ replacement: teeth are easily accessible, and whereas our quality of life is greatly improved if we have them, we do not need our teeth to live. These may seem trivial points, but as the first wave of replacement organs start to make their way toward the clinic, teeth will serve as a crucial test of the feasibility of different tissue engineering techniques. With organs essential to life, doctors will have no leeway to make mistakes, but mistakes with teeth would not be life-threatening and could be corrected.

This is not to say that engineering teeth will be simple. Millions of years of evolution went into establishing the complex processes that produce organs, teeth included, during embryonic development. The challenge for tissue engineers is to rep-

licate those processes, which are tightly controlled by the growing embryo's genes. A good way to start learning how to build teeth, therefore, is to observe how nature does it.

Delicate Dialogue

JUST SIX WEEKS after conception, a human embryo is less than an inch long and barely beginning to take recognizable shape. Yet a constant cross talk among its cells is already initiating and guiding the formation of its teeth. The intricacy of such signal exchanges is among the reasons that teeth and other organs cannot as yet be grown entirely in dishes in laboratories. Indeed, scientists may never be able to completely reproduce these conditions artificially. The more we understand these early developmental processes, however, the greater will be our chances of providing engineered tooth tissues with the most important cues for organ building and letting nature do the rest.

Most organs, for example, arise through interactions between two distinct embryonic cell types, epithelial and mesenchymal, and teeth are no exception. In the embryo, oral epithelial cells (which are destined to line oral cavities) send out the first inductive signals to mesenchymal cells (which will produce jawbone and soft tissues), instructing them to begin odontogenesis, or tooth formation. Once the mesenchymal cells have received their initial instructions, they start sending signals back to the epithelial cells. This reciprocal exchange continues throughout embryonic tooth development.

At first, the future tooth is no more than a thickening in the embryonic oral epithelium. As it grows, the epithelium begins to penetrate the underlying mesenchymal tissue, which in turn condenses around the protrusion, forming a tooth bud by the embryo's seventh week [see box on opposite page]. As the epithelium penetrates farther, it wraps itself around the condensing mesenchyme, eventually forming a bell-shaped structure, open at its bottom, around 14 weeks. Ultimately, the epithelium will become the visible outer enamel of the tooth that erupts from the baby's gum line some six to twelve months after birth, and the mesenchymal cells will have formed the nonvisible parts of the tooth, such as dentin, dental pulp, cementum, and a periodontal ligament that attaches the tooth to the jawbone.

Even before this tooth begins forming, its shape will be predetermined by its position. Some of the same epithelial signals that trigger initiation of odontogenesis also regulate

Overview/Cutting-Edge Teeth

- Tissue engineers working toward creating living replacement teeth take cues from nature as they coax disparate cell types to form a functional organ.
- Alternative methods include building teeth from existing dental cells or growing them from progenitor tissues. Both approaches have already produced structurally correct teeth.
- Remaining challenges include growing roots and identifying ideal raw materials for bioengineered human teeth, but progress has been rapid and test-tube teeth may become the first engineered organs.

HOW NATURE ENGINEERS A TOOTH

It may look simple from the outside, but on the inside a tooth is a tiny marvel of design and construction that takes about 14 months to complete in a developing human. Two different types of primordial embryonic tissue combine to produce a tooth,

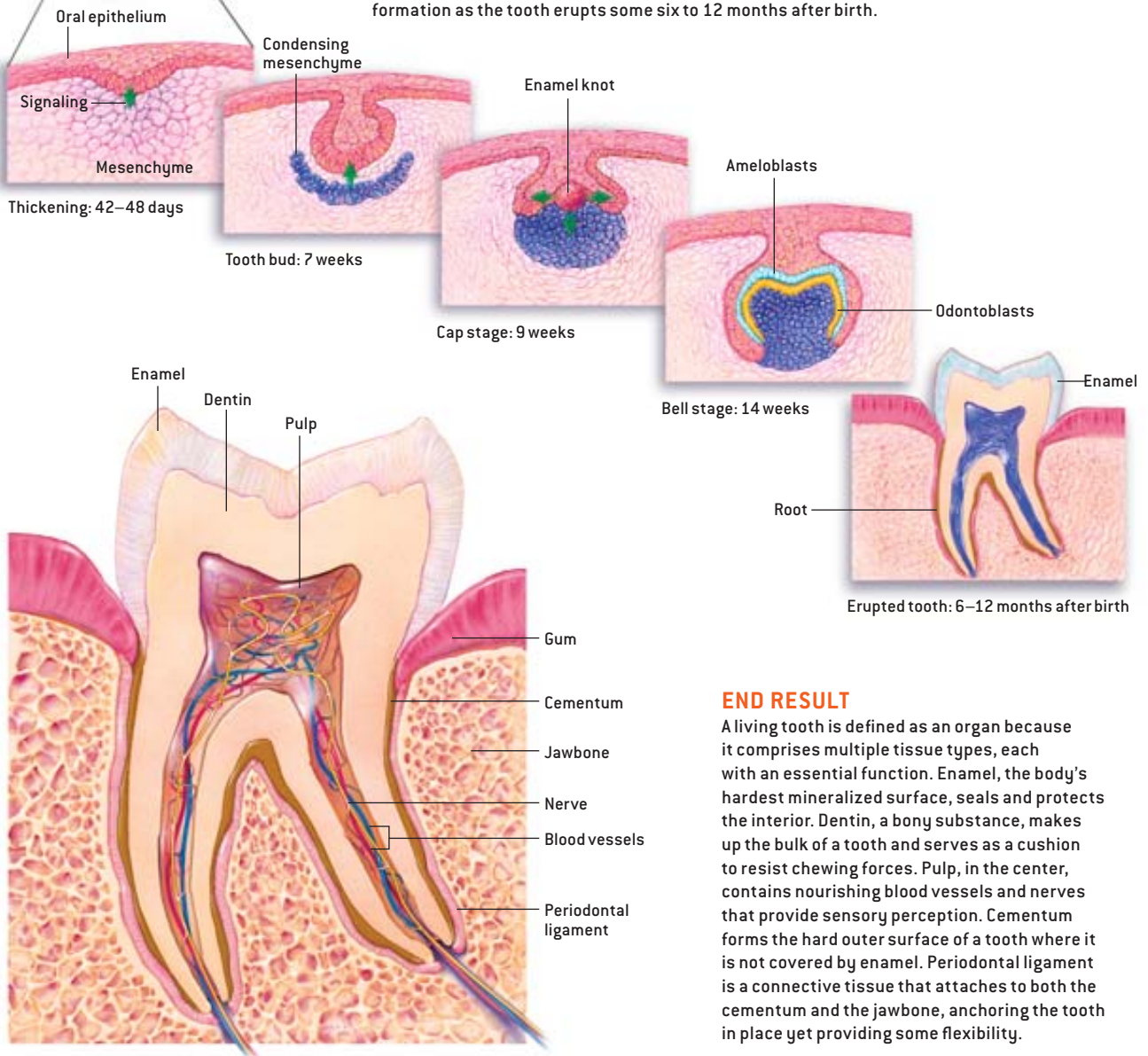
and an ongoing molecular dialogue between them directs the process. Tissue engineers are studying these signals and steps to understand the cues they need to replicate as they create living bioengineered replacement teeth.

Embryo at 6 weeks



TOOTH FORMATION

Just six to seven weeks into human embryonic development, when the whole head is still taking shape, teeth are also beginning to form. At the location of a future tooth, oral epithelial tissue thickens slightly and gene activity within its cells causes signals to be sent to underlying mesenchymal tissue. As the epithelium penetrates farther, mesenchymal cells respond by emitting their own signals and condensing around the protrusion to form a tooth bud. By week nine, the epithelium has become a cap atop condensed mesenchyme. A structure at its center called the enamel knot is now a primary source of signals directing the activity of both epithelial and mesenchymal cells. At 14 weeks, the tooth germ has a bell shape comprising differentiating cells called ameloblasts, which will later become enamel, and odontoblasts, which will form dentin. Roots are the last structures to develop, completing their formation as the tooth erupts some six to 12 months after birth.

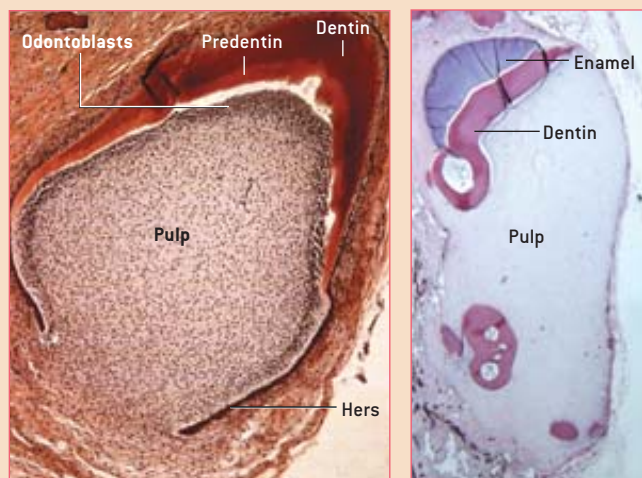
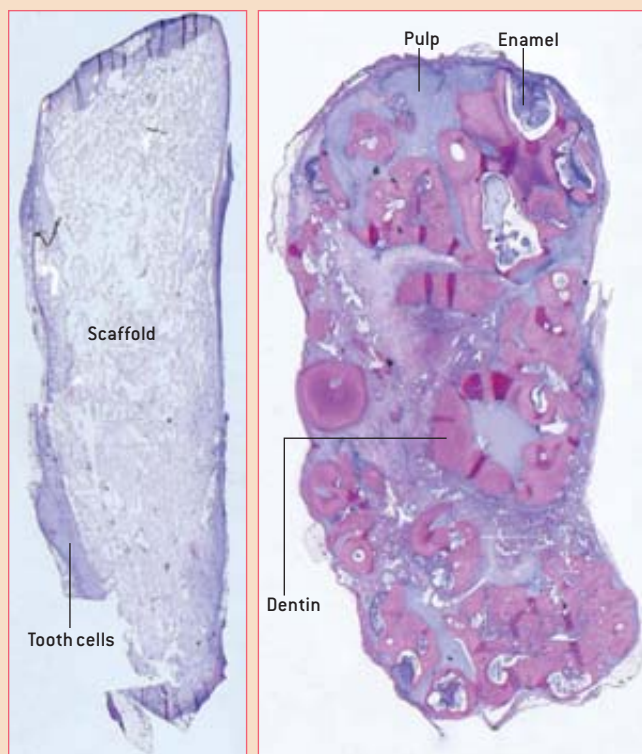


END RESULT

A living tooth is defined as an organ because it comprises multiple tissue types, each with an essential function. Enamel, the body's hardest mineralized surface, seals and protects the interior. Dentin, a bony substance, makes up the bulk of a tooth and serves as a cushion to resist chewing forces. Pulp, in the center, contains nourishing blood vessels and nerves that provide sensory perception. Cementum forms the hard outer surface of a tooth where it is not covered by enamel. Periodontal ligament is a connective tissue that attaches to both the cementum and the jawbone, anchoring the tooth in place yet providing some flexibility.

Cells Reunite to Form Teeth

Tooth cells taken from adolescent pigs and seeded onto a biodegradable scaffold are visible in blue along its edges after one week of incubation (*top left*). Following 25 weeks of growth (*top right*), the scaffold has dissolved and new dental pulp, enamel, and dentin have taken its place. In a series of such experiments, tiny toothlike structures grew amid the new tissues. Correct tooth-tissue organization (*bottom left*), including a pre-root structure known as Hertwig's epithelial root sheath (Hers), was observed in 15 to 20 percent of the miniature teeth. In other instances, the tooth structure was incorrect or incomplete (*bottom right*). These bioengineered teeth nonetheless seem to confirm that disaggregated dental cells can reorganize themselves into larger dental tissues.



an important category of genes in the jaw mesenchyme. Known as homeobox genes, they participate in determining the shape and location of organs and appendages during embryonic development throughout the body. In a developing human jaw, different homeobox genes are activated in different areas, guiding each tooth bud down a pathway to become a molar, premolar, canine or incisor.

A homeobox gene called *Barx1*, for example, is switched on, or expressed, by mesenchymal cells in the positions where molar teeth will grow. In animal experiments, causing *Barx1* to be misexpressed in mesenchyme that would normally form incisors makes those teeth develop with a molar shape instead. Because the ability to predict and control tooth shape will be essential for the creation of engineered teeth, scientists can use the activity of genes such as *Barx1* as definitive predictive markers of future shape when teeth created in the lab are first growing in culture.

In turn, we must provide the right signals to the developing teeth at the right time. As early as the 1960s, researchers such as Shirley Glasstone of Strangeways Research Laboratory in Cambridge, England, began exploring the possibility of growing teeth by experimenting with mouse tissues. In seminal studies performed over the next three decades, tiny pieces of embryonic mouse dental epithelium and dental mesenchyme were brought together and then either grown in a tissue culture dish or surgically implanted in the body of a host where the recombined tissues would receive a blood supply. These experiments demonstrated that such embryonic tooth primordia could continue to develop as if they were still in the embryo, producing dentin and enamel. Their development arrests early, however, and they do not ultimately yield fully formed teeth. Something is missing from their environment.

The growth factors and other signals required to complete tooth formation in an embryo most likely come from surrounding jaw tissue. Thus, transplanting tooth primordia into the jaw to finish developing would seem to be a simple solution. When replacement teeth are engineered, for instance, they will ideally be grown in their permanent location so that they can create nerve and blood vessel connections and physically attach themselves to the jawbone. The adult jaw is a vastly different environment from the embryonic version, however, and scientists have been unsure whether it would provide the correct signals to a developing tooth.

Moreover, tooth primordia must be constructed from the right combination of cells to reproduce natural tooth material and structure. Being able to use cells from a patient's own body would be preferable to using embryonic cells because the patient's own tissue would not be perceived as foreign and so would not provoke an immune response.

Three key milestones must therefore be reached to establish whether engineering replacement biological teeth is possible. Sources of cells that can form teeth and are easily obtained from patients themselves must be identified. The teeth produced from these cells must be able to develop in the environment of the adult jaw, producing roots that are attached to

Each cell seems to know its place in the larger collective.



the bone by a functional periodontal ligament. And the shape and size of these biological teeth must be predictable and controllable so that they can be made to match the patient's own teeth. These are ambitious goals, but considerable progress toward each is being made by different research groups using somewhat disparate approaches.

Building Bioteeth

IN THE LATE 1980S organ transplant surgeon Joseph P. Vacanti of Harvard Medical School and polymer chemist Robert S. Langer of the Massachusetts Institute of Technology conceived the idea of placing the cells of an organ or tissue on a prefabricated biodegradable scaffold with the goal of generating tissues and organs for transplantation [see "Artificial Organs," by Robert S. Langer and Joseph P. Vacanti; *SCIENTIFIC AMERICAN*, September 1995]. In simplified terms, their approach was based on the fact that living tissues are made of cells constantly signaling to one another and often moving around within a three-dimensional community of sorts. Each cell seems to know its place and role in the larger collective that forms and maintains a functional tissue. Therefore, if the right mix of dissociated cells is reaggregated within a scaffold that replicates their natural 3-D environment, the cells should instinctively reform the tissue or organ to which they belong.

Vacanti and Langer's early successes regenerating pieces of liver tissue from liver cells using this scaffold-based strategy have since led to widespread experimentation with the technique to produce other complex tissues, such as heart muscle, intestine, mineralized bone and now teeth. Pamela C. Yelick and John D. Bartlett of the Forsyth Institute in Boston began working with Vacanti in 2000 to investigate the feasibility of engineering teeth this way by focusing on pigs, which, like humans, produce two sets of teeth over their lifetime.

One of us (Young) also took part in these experiments for which raw material was derived from the unerupted third molars ("wisdom teeth") of six-month-old pigs. To obtain a heterogeneous random mixture of dental enamel epithelial and pulp mesenchymal cells, the pig teeth were broken into tiny pieces and then further dissolved using enzymes. Tooth-shaped scaffolds were made from biodegradable polyester plastics and coated with a substance that makes the plastic sticky so cells can adhere to it. The cell mixtures were seeded into the scaffolds, and the constructs were surgically implanted into rat hosts, wrapped in omentum, a fatty white material rich in blood vessels that surrounds the intestines. This step is

important because the developing tooth tissues require an ample blood supply to provide them with nutrients and oxygen while they grow.

Initially the scaffolds provided support for the cells, but later they dissolved as intended and were replaced by new tissue. When the implants were examined after 20 to 30 weeks, tiny toothlike structures were visible within the confines of the original scaffold. Their shape and the organization of their tissues resembled the crowns of natural teeth [see *box on opposite page*]. They also included most of the tissues that make up a normal tooth, demonstrating for the first time that enamel, dentin, pulp, and features that appeared to be developing tooth roots could be regenerated on scaffolds.

It seemed that mixtures of dental cells could reorganize themselves on scaffolds into arrangements that favor formation of mineralized enamel, dentin and soft tooth tissue. Another possible explanation for these exciting results, of course, was that the random arrangement of cells seeded onto the scaffold favored tooth tissue development only by chance. The Forsyth group therefore tested these possibilities in a new study using dental epithelial and mesenchymal cells isolated from the first, second and third molars of rats. This time, however, the cells were grown and their numbers expanded in tissue culture for six days before their being seeded onto scaffolds and implanted in rat hosts. After 12 weeks' growth, the resulting tissues were extracted and examined. Once again, small tooth structures consisting of enamel, dentin and pulp tissue were observed to have formed within the original scaffold.

These new results were encouraging because they lent some weight to the previous evidence that cells can reorganize them-

THE AUTHORS

PAUL T. SHARPE and CONAN S. YOUNG met two years ago at a tooth and bone conference where they discovered a shared fondness for mountain biking and soccer (one calls it "football"), despite their differing approaches to bioengineering teeth. Sharpe established and heads the department of craniofacial development at Guy's Hospital in London and is also Dickinson Professor of Craniofacial Biology at King's College London. In 2002 he founded Odontis Ltd., a biotechnology company devoted to growing human teeth and bone by emulating their formative processes in a developing embryo. Young is an instructor in oral and developmental biology at the Harvard School of Dental Medicine and a staff scientist at the Forsyth Institute in Boston, where he is working toward growing teeth from cells seeded onto biodegradable scaffolds.



No one knew whether the adult jaw would provide signals for teeth to form.

selves into tooth-forming configurations. Moreover, the cells did not appear to have been adversely affected by being expanded in culture—a process that will be essential in engineering human replacement teeth because tissue engineers would probably have to craft a replacement tooth from small samples of the patient's own cells. And, finally, the experiment demonstrated that tooth regeneration is possible in a second mammal, making the success of a similar approach in humans more likely.

Although the Forsyth team was able to generate most of the desired tissue types with cells from an adult source, those tissues organized themselves into the proper arrangement for a natural tooth only 15 to 20 percent of the time. The group is therefore continuing to work on methods of more precisely placing different dental cell types within scaffolds to achieve a more accurate tooth structure.

At the same time, the team is exploring the possibility that the new tooth tissues observed in these experiments might not have been produced solely by reorganization of the dissociated dental cells. Instead the third molar tooth buds that provided cells to seed the scaffolds might have contained hidden stem cells—potent progenitors of other cell types—that were responsible for forming the new tissue. If true, this would mean that new dental stem cells capable of producing nearly all the dental tissue types required for bioengineering teeth might exist within teeth themselves, at least until early adulthood, when wisdom teeth erupt. Such versatile adult dental stem cells would certainly speed efforts to generate teeth on scaffolds, and they might also facilitate the tooth-engineering approach used by the Sharpe group at King's College London.

Teeth from Scratch

RATHER THAN ATTEMPTING to build adult teeth from their constituent cells, one of us (Sharpe) is pursuing a strategy based more closely on reproducing the natural processes of embryonic tooth development described earlier. In essence, the method requires an understanding of the basic principles controlling early tooth formation and a source of cells to play the roles of embryonic oral epithelium and mesenchyme.

To date, the Sharpe group has experimented primarily with mouse cells, using both stem cells and ordinary cells, from embryonic as well as adult sources, to test the potential of various cell types to produce replacement teeth. In most cases, the group began by aggregating mesenchymal cells in a centrifuge until they formed a small solid mass. This pellet was

then covered in epithelium and cultured for several days, while the gene activity in its tissues was monitored for indications of early tooth development. Next, these tooth primordia were implanted into the bodies of animal hosts in locations where they could receive a nourishing blood supply, such as the kidney of a mouse, and left to grow for about 26 days.

In the course of these experiments, clear tooth formation was observed but only when the epithelium came from an embryonic source and the mesenchymal cell populations contained at least some stem cells. When stem cells from adult bone marrow took the place of oral mesenchyme, for example, the transplanted constructs produced structurally correct teeth. Thus, it seems embryonic mesenchyme can be replaced with adult stem cells to generate new teeth.

Unfortunately, many years of experiments have established that embryonic epithelium contains a unique set of signals for odontogenesis that disappear from the mouth after birth. The Sharpe group is continuing to seek an effective population of substitute cells that could be derived from an adult source. Still, the results achieved with primordia made from the combination of adult stem cells and embryonic oral epithelium have been extremely encouraging.

Significantly, these teeth were also in the normal size range for mouse teeth, they were surrounded by new bone and connective tissue, and they showed the earliest signs of root formation. The next step was to see whether such explants could also form teeth in the mouth. In the embryonic jaw, soft tissues, teeth and bone are all developing together without external stresses such as chewing and talking, whereas the adult jaw is a hard, busy place. No one knew whether it would provide the necessary signals for teeth to form and integrate themselves into the environment as they would in an embryo.

To find out, the Sharpe group extracted tooth buds from embryonic mice, then transplanted them into the mouths of adult mice. Small incisions were made in the soft tissue of the upper jaw of the host mice, in a region known as the diastema between the molars and incisors where normally there are no teeth. The embryonic tooth primordia were inserted into these pockets and sealed in place with surgical glue. Afterward, the mice were fed a soft diet and the transplants monitored. Just three weeks later teeth could be clearly identified in the diastema. They had formed in the correct orientation, were of appropriate size for the mice, and were attached to underlying bone by soft connective tissue [see illustration on opposite page].

Remarkably, it appears that the adult mouth can provide a suitable environment for tooth development. That is just one of the three milestones toward engineering replacement teeth that we identified earlier, however. The road to human bioengineered teeth may yet have a few twists.

On the Cusp

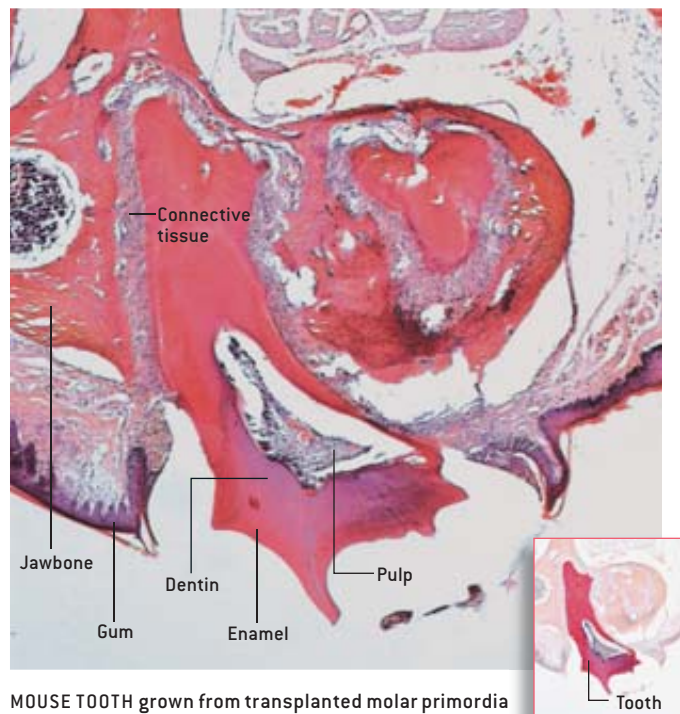
COMPARED WITH EFFORTS to engineer other organs, teeth have made considerable progress in a short time. The overall challenge remains developing methods that are simple yet controlled.

Another of the targets that we established, the ability to predict and control tooth size and shape, is close. In cultured primordia, molar and incisor tooth germs can easily be distinguished by their appearance and their gene activity, although other shapes found in the human mouth, such as premolars and canines, are more difficult.

The teeth grown from embryonic primordia in the mouths of adult mice by the Sharpe group displayed shapes appropriate to their original locations in the embryo—molar primordia grew into molar-shaped teeth, for example. Because shape signals are received at the very start of natural tooth development, the embryonic tooth germs were already programmed. Tissue engineers need to better understand these initial shape signals to induce them in human bioteeth.

To date, the teeth generated by any of the tissue engineering methods we have described have not developed roots. In truth, both root development and the stimuli that initiate tooth eruption are complex and still little understood. Roots are the last part of teeth to form, completing their development during the eruption process, and more research is needed to understand what conditions would best favor their creation in replacement teeth. Another unknown is how long engineered human teeth would take to fully form in an adult mouth. Humans' second set of "adult" teeth also begins developing in the embryo, yet those teeth take six to seven years to finally erupt—or 20 years in the case of wisdom teeth. Our experience with tooth generation in animals suggests that an engineered human tooth would form far more quickly, but we do not know if it might take longer to fully mature and its enamel to completely harden.

Of course, most research into bioengineered tooth production is also working toward finding an effective and easily accessible source of the patient's own cells to use as raw material. Immune rejection would be avoided, and because tooth size, shape and color are genetically determined, the engineered teeth would more closely match the patient's natural teeth. The Sharpe group has found that adult mesenchymal stem cells derived from bone marrow (but also possibly obtainable from fat) can replace embryonic mesenchyme in the tooth formation process. A substitute for embryonic epithelium has yet to be identified, although purported adult stem cells have been discovered in other tissues with epithelial origins, such as skin and hair. These or some other adult cell type may prove effective, perhaps with the aid of gene manipulation



MOUSE TOOTH grown from transplanted molar primordia in the upper jaw of a host mouse demonstrates that new teeth can develop in the adult mouth. The tooth at center in this cross section of the jaw's diastema region has broken through the gum line (a second tooth above it and to the right is still forming). Pulp is visible inside the emerged tooth. Red stain colors dental hard tissues, highlighting enamel and dentin. Although lacking roots, the tooth is attached to surrounding jawbone by soft connective tissue.

to induce the appropriate initiating signals for odontogenesis.

Of the several potential cell sources, teeth themselves may be the most convenient. The Forsyth group's results suggest that stem cells capable of forming tooth tissues, including enamel, could be present within teeth. Researchers elsewhere have also shown that dentin and other tooth tissues experience some natural regeneration after injury, which, too, suggests the presence of progenitor cells capable of generating a variety of tooth tissues. Thus, the possibility exists of someday soon fashioning new teeth from old.

MORE TO EXPLORE

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